Short communication: Molecular typing of *Prototheca zopfii* from bovine mastitis in Japan

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ABSTRACT

Prototheca zopfii causes bovine mastitis, resulting in reduced milk production and the secretion of thin watery milk with white flakes. *Prototheca zopfii* has been biochemically and serologically divided into at least 2 genotypes, *P. zopfii* genotype 1 and *P. zopfii* genotype 2. The latter is known to be the main causative agent of bovine protothecal mastitis. *Prototheca zopfii* was later reclassified into 5 varieties: var. *zopfii* (genotypes 1 and 2), var. 1 (formerly *Prototheca blaschkeae*), var. 3 (formerly *P. moriformis*), and var. *portoricensis*. In this study, the 18S ribosomal DNA sequences of diverse clinical specimens from different areas in Japan were studied to clarify the pathogenicity of *P. zopfii* var. *zopfii*. The phylogenetic tree revealed that all genotype 2 isolates were grouped in a cluster of *P. zopfii* var. *zopfii* SAG 2021^T (type strain genotype 2), and were independent from the cluster of the genotype 1 isolates. Thus, all isolates from bovine mastitis in Japan were identified as *P. zopfii* genotype 2. Therefore, *P. zopfii* var. *zopfii* genotype 2 is associated with bovine mastitis. **Key words:** bovine *Prototheca* mastitis, genotype, *Prototheca zopfii*, 18S ribosomal DNA

Short Communication

The genus *Prototheca* consists of achlorophyllic algae that are ubiquitous among cow-barn surroundings. *Prototheca zopfii* is associated with bovine mastitis, which causes a reduction in milk production and the secretion of thin, watery milk with white flakes. *Prototheca zopfii* has been biochemically and serologically divided into at least 2 genotypes, *P. zopfii* genotype 1 and *P. zopfii*

genotype 2 (Roesler et al., 2003, 2006). Isolates from bovine mastitis in Germany, Italy, Japan, Portugal, and Poland were almost all identified as *P. zopfii* genotype 2, suggesting that it is the main causative agent of bovine mastitis (Roesler et al., 2003, 2006; Möller et al., 2007; Marques et al., 2008; Osumi et al., 2008; Ricchi et al., 2010; Jagielski et al., 2011). Afterward, Pore (2011) reclassified *P. zopfii* into 5 varieties: var. *zopfii* (genotypes 1 and 2), var. 1 (formerly *Prototheca blaschkeae*), var. 3 (formerly *P. moriformis*), and var. *portoricensis*. However, further studies on the molecular typing of *P. zopfii* var. *zopfii* (genotypes 1 and 2) are needed to ascertain its genetic characteristics as well as its etiologic role in bovine mastitis. In this study, the 18S ribosomal (r)DNA sequences of diverse clinical specimens from different areas in Japan were studied to clarify the pathogenicity of *P. zopfii* var. *zopfii*.

The *P. zopfii* type strains of *P. zopfii* var. 1 (SAG2064^T), genotype 1 (SAG2063^T), and genotype 2 (SAG2021^T) were used for this study. In addition to the type strains, 1 isolate of *P. zopfii* var. 1 and 21 clinical strains of *P. zopfii* were isolated from 21 cases of bovine (Holstein cow) mastitis from the Aichi, Chiba, and Hokkaido areas in Japan (Table 1). One strain of *P. zopfii* var. 1 and 10 strains of genotype 1 were isolated from farm stock in the Aichi area (Table 1), as described in our previous study (Osumi et al., 2008; Kishimoto et al., 2010).

All strains were grown on Sabouraud dextrose agar at 27°C for 1 wk, and were then collected by scraping and suspended at 37°C for 14 h in lysis buffer [1 mg/mL of zymolyase 100T (Takara, Kyoto, Japan), 0.1 mM EDTA, 1% SDS, 10 mM Tris hydrochloride (pH 8.0), and 0.3% 2-mercaptoethanol].

The primer set for the protothecal 18S rDNA domain was designed by Roesler et al. (2006) as follows: Proto 18S-4F (5'-GACATGGCGAGGATTGACAGA-3') and Proto 18S-4R (5'-AGCACACCCAATCGGTAGGA-3'), where F is forward and R is reverse.

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Table 1. *Prototheca* isolates in Japan used in this study

Variety	Strain	Origin	Genotype ¹
var. 1	O2	Bovine feces	
var. <i>zopfii</i>	NUBS 1	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 24	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 26	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 28	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 44	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 69	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 89	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 101	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 114	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	NUBS 118	Bovine mastitis in Aichi	2
var. <i>zopfii</i>	T1	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T2	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T3	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T4	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T5	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T6	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T7	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	T8	Bovine mastitis in Chiba	2
var. <i>zopfii</i>	B397	Bovine mastitis in Hokkaido	2
var. <i>zopfii</i>	D397	Bovine mastitis in Hokkaido	2
var. <i>zopfii</i>	D455	Bovine mastitis in Hokkaido	2
var. <i>zopfii</i>	NUBS 73	Sewage	1
var. <i>zopfii</i>	NUBS 65	Bovine feces	1
var. <i>zopfii</i>	NUBS 68	Bovine feces	1
var. <i>zopfii</i>	NUBS 74	Bovine feces	1
var. <i>zopfii</i>	NUBS 82	Bovine feces	1
var. <i>zopfii</i>	NUBS 88	Bovine feces	1
var. <i>zopfii</i>	NUBS 21	Drinking water	1
var. <i>zopfii</i>	NUBS 41	Drinking water	1
var. <i>zopfii</i>	NUBS 70	Drinking water	1
var. <i>zopfii</i>	NUBS 117	Drinking water	1

¹Identified by 18S ribosomal DNA sequence analysis.

Thirty-five cycles of PCR amplification were performed under the following conditions: denaturation for 30 s at 95°C, primer annealing for 1 min at 58°C, and polymerization for 1 min at 72°C in a total reaction volume of 20 µL of amplification mixture [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin, 200 mM deoxynucleotide triphosphate, 1.0 U of *Taq* polymerase (Takara), and 0.5 µg of each primer]. The resultant amplified DNA fragments were electrophoresed on a 2% (wt/vol) agarose gel with 1× Tris-acetate-EDTA buffer and visualized by ethidium bromide.

A DNA band of approximately 450 bp for each strain was excised from the gel and then purified with an ExoSAP-IT kit (USB Corporation, Cleveland, OH) and sequenced on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The resulting sequences were compared with those of the type strains and isolates (Table 1) by using the ClustalW multiple-sequence alignment program (Thompson et al., 1994). The phylogenetic tree was constructed by the neighbor-joining method using the TreeView program (Page, 1996). Bootstrap analysis

was performed on 100 random samples and analyzed by the ClustalW program (Felsenstein, 1985).

This study was performed to clarify the phylogenetic relationships among the genotypes of *P. zopfii* var. *zopfii* and other varieties of *P. zopfii*. The phylogenetic analysis of clinical isolates based on the 18S rDNA sequences revealed that these isolates were grouped in a cluster of *P. zopfii* var. *zopfii* SAG 2021^T (type strain genotype 2) and *P. zopfii* var. 3 ATCC 50081 (GenBank accession number AB077048), and were independent from the cluster of the genotype 1 isolates (Figure 1). *Prototheca zopfii* var. 3 is differentiated from var. *zopfii* by capsule production and has been regarded as non-pathogenic (Pore, 2011). In this study, we sequenced a type strain (SAG2064^T) and 1 clinical isolate of *P. zopfii* var. 1 (formerly *P. blaschkeae*). The phylogenetic analysis of 18S rDNA sequences also indicated that *P. zopfii* var. 1 was genetically distinct from the other *P. zopfii* varieties. Therefore, *P. zopfii* var. 1 should be classified as a different species (formerly *P. blaschkeae*) from *P. zopfii*.

The resulting sequences were also aligned, and the phylogenetic tree was constructed by maximum par-

Figure 1. A phylogenetic tree by the neighbor-joining method showing the molecular taxonomy of 18S ribosomal gene fragments. The numbers at the branches were determined by the bootstrap value indicating the times in 100 repeat subsamples in a monophyletic grouping. Items in parentheses indicate the GenBank accession number of the 18S ribosomal gene of algae species.

simony using the program MEGA 5.1 (<http://www.megasoftware.net/>). The phylogenetic tree created by maximum parsimony also indicated the neighbor-joining tree constructed from the current study (Figure 2).

Recently, genotypes 1 and 2 were reclassified as the same variety of *P. zopfii* (Pore, 2011). In our previous report, we examined the susceptibility of *P. zopfii* genotype 1 and 2 isolates to amphotericin B, gentamicin,

Figure 2. A phylogenetic tree by the maximum parsimony method showing the molecular taxonomy of 18S ribosomal gene fragments. Items in parentheses indicate the GenBank accession number of the 18S ribosomal gene of algae species.

kanamycin, and itraconazole by *E*-test (Sobukawa et al., 2011). *Prototheca zopfii* var. *zopfii* genotype 1 was more susceptible to amphotericin B, gentamicin, and kanamycin than was *P. zopfii* var. *zopfii* genotype 2 (Sobukawa et al., 2011). These 2 genotypes are distinct in phenotype, pathogenicity, and 18S rDNA sequence. Therefore, *P. zopfii* var. *zopfii* remains to be reclassified at the variety level. The reservoir of the etiologic agent of bovine mastitis is thought to be the environment because *P. zopfii* is often found in flowing and standing water. However, these environmental isolates belong to

genotype 1, whereas clinical isolates from bovine mastitis were identified as genotype 2. Further molecular typing of *P. zopfii* var. *zopfii* (genotypes 1 and 2) needs to be completed to identify preventative measures for bovine mastitis.

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